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Mutations in genes that regulate the cell cycle are the most common genetic changes in cancer cells. P27kip1 is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors. Decreased p27 expression is found in a wide variety of human neoplasms and is associated with poor patient outcome in many human cancers. Although these studies of p27 expression in primary tumors have correlated low p27 expression with poor prognosis, they do not demonstrate that p27 loss is a causal step in tumorigenesis. In mice however, p27 functions as a tumor suppressor that exhibits haplo-insufficiency.

The goal of this research was to investigate the role of p27 in tumorigenesis by characterizing mechanisms of p27-associated tumorigenesis, and identifying proteins that cooperate with p27-loss during tumorigenesis. Using insertional mutagenesis, we identified several novel and previously known oncogenes that cooperate with p27-loss during lymphomagenesis. We also examined the interaction of p27-loss with mutations within the Wnt pathway in mice, but failed to observe synergy during mammary carcinogenesis. Finally, we specifically investigated the hypothesis that p27 and p130, a member of the Retinoblastoma protein family, cooperate in tumor suppression. However, our studies did not reveal cooperativity between these two proteins with respect to tumorigenesis.

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Introduction

Mutations in genes that regulate the cell cycle are the most common genetic changes in cancer cells. One gene that has been intensely studied is p27kip1, a member of the Cip/Kip family of cyclin-dependent kinase inhibitors. Decreased p27 expression is found in a wide variety of human neoplasms and is associated with poor patient outcome in many cancers, including carcinomas of the breast, colon, lung and prostate, and lymphoma. Although these studies of p27 expression in primary tumors have correlated low p27 expression with poor prognosis, they do not demonstrate that p27-loss is a causal step in tumorigenesis. Studies in p27 knockout mice, however, have directly implicated p27 as a dosage-dependent tumor suppressor. Mice with targeted p27 deletions develop pituitary adenomas with high penetrance, and they are hypersensitive to radiation and chemical carcinogenesis. Furthermore, tumor suppression by p27 in mice is haplo-insufficient (e.g. the tumor incidence in p27 heterozygotes is intermediate to that of p27-null or wild type animals), suggesting that the decreased levels of p27 expression observed in human cancers may have similar consequences. Despite its well-studied function as an inhibitor of cyclin-dependent kinases, the mechanism of tumor suppression by p27 is not clearly understood. The goal of this research was to investigate the role of p27 in tumorigenesis by characterizing mechanisms of p27-associated neoplasia, and identifying proteins that cooperate with p27-loss in multi-step tumorigenesis.

Body

Task 1: Identification of genes that cooperate with p27 in multistep tumorigenesis. The goal of this Aim was to use retroviral insertional mutagenesis to identify genes that cooperate with p27-loss during tumorigenesis. As detailed and approved in our previous reports, this task was removed from this project after independent funding was obtained. However, because the initial phase of this research was supported by this award, we have referenced the award on a recent submitted manuscript, and have summarized this work below.

We Moloney murine leukemia virus (M-MuLV) to study lymphomagenesis because it is a potent carcinogen in most strain backgrounds, and because many insertion sites have been previously identified. Approximately 100 newborn mice from p27+/- X p27+/- crosses were infected i.p. with MuLV. In this initial study, the latency of lymphomas induced by M-MuLV was strongly dependent upon p27-genotype, and the p27 null animals developed lymphomas and died within a remarkably narrow window approximately 20 weeks after infection. Thus like radiation and chemical carcinogens, p27-null mice develop retroviral-associated neoplasms at an increased rate.

We used three approaches to identify common insertion sites in M-MuLV-induced lymphomas. In the first approach we directly screened lymphoma DNAs by Southern blotting for involvement of previously defined M-MuLV common insertion sites. The second two approaches used inverse PCR (IPCR) to clone genomic DNA fragments immediately adjacent to proviruses. The IPCR clones were sequenced, and individual sites were analyzed to determine if they represented common insertion sites using two strategies. In the second approach, clones with sequences that did not correspond to known common insertion sites were used as probes to examine independent p27-null and wild type lymphomas for insertions within these loci by Southern blotting, The final approach relied on the recent completion of the Celera mouse genome. Sequenced IPCR clones were searched against the Celera database and clones that fell within the same Celera "scaffolds" (contigs) were examined further to precisely map the insertion sites.

We found that insertions leading to activation of myc genes occurred more often in p27-null animals, thus identifying myc activation as an event that may cooperate with p27-loss in lymphomagenesis. We also identified two novel loci that were frequently activated in p27-null lymphomas: The first are insertions within the jun dimerization protein 2. This is the first report that jdp-2 functions as a proto-oncogene. Several lines of evidence support the identification of jdp-2 as the target gene of the insertions within this region of chromosome 12: 1) most insertions fell directly within the jdp2-gene, 2) insertions in cluster A result in increased jdp-2 mRNA and protein expression, 3) insertions within cluster B produce chimeric viral/jdp-2 transcripts initiating in the viral LTR which are the hallmark of promoter insertion, and 4) expression of the nearby c-fos gene is unaffected by insertions within either cluster. Many of the integrations caused truncation of the jdp-2 protein. However, the fact that several of the lymphomas that we mapped contained insertions that leave the jdp-2 coding sequence intact indicates that truncation is not required for jdp-2 activation.

The X-liked XPC-1 locus is the second novel common insertion site that we found to be enriched in p27-null lymphomas. Integrations within this locus are tightly clustered, strongly suggesting a common target gene. However, thus far neither our cloning efforts nor the use of genomic informatics has clearly defined the target of these insertions. We have obtained a phage clone corresponding to this locus and analyzed a candidate transcript corresponding to a mouse EST sequence which is contained within this clone and overexpressed in all of the lymphomas containing XPC-1 insertions. There is significant sequence conservation between the murine XPC-1 locus and the syntenic human Xq26 region, and sequences homologous to AI464896 and the cloned insertion sites are present in the human Xq26 region with spacing quite similar to the murine locus. However, AI464896 does not contain an open reading frame. Three possible explanations are: 1) this insertionally activated EST may function as a non-coding RNA,

2) this transcript may not be the true target of the insertion cluster, or 3) it may represent only a partial cDNA

Finally in collaboration with Anton Berns (Amsterdam) we have completed a large-scale analysis of 350 cloned insertion sites that have been compared with the Celera mouse genome database. We have now identified several additional novel candidate p27-complementing loci. This analysis revealed a remarkable number of common insertion sites within this defined group of lymphomas, and illustrates the power of high throughput insertional mutagenesis in cancer gene discovery.

Task 2: Determination of Tumor Susceptibility in p27 and p27/p130 -/- double knockout mice. At least three major studies using different and large patient populations have shown that reduction of p27 protein expression in breast tumors is associated with significantly reduced patient survival. However, the lack of mutations in the gene that encodes p27 in tumors has made it difficult to establish causality between reduction of p27 protein expression and tumor progression. We previously demonstrated that p27 deficient mice were predisposed to chemically induced tumor development in multiple tissues including skin, lung, small intestine, colon, ovary and uterus, demonstrating that p27 functions as a tumor suppressor. The objective of this aim was to determine if p27 deficient mice were predisposed to breast tumor development.

A cohort of *p27+/+*, *p27+/-*, and *p27-/-* mice on a 129/Sv genetic background were treated with the mammary carcinogen dimethylbenzanthracene (DMBA) by gavage (1 mg, once per week for 6 weeks). Unexpectedly, all the *p27-/-* mice succumbed to pituitary tumors between 3-4 months of age. However, 75% (3/4) of these mice had extensive mammary tissue hyperplasia at the time of sacrifice. *p27+/-* and wild type mice developed breast tumors, but after a much longer latency. Thus the early development of DMBA-induced pituitary tumors in *p27-/-* mice precluded determination of breast cancer risk in these mice. Nevertheless, the extensive mammary tissue hyperplasia observed in 75% of the *p27-/-* mice suggests that p27 deficiency may accelerate breast tumor development.

To circumvent the interference of DMBA-induced pituitary tumorigenesis in *p27* null mice we crossed p27 deficient mice to MMTV- *Wnt-1* transgenic mice that spontaneously develop breast tumors. These mice express the *Wnt*-1 oncogene in the mammary epithelia and 100% of female mice develop adenocarcinoma of the breast by 40 weeks of age. We generated *Wnt*-1 transgenic mice with all three *p27* genotypes: *p27*+/+, +/-, and -/- to measure the effect of p27 reduction on breast tumor latency. We set up a similar experiment on two genetic backgrounds, pure C57BL6 and C57BL6 x 129 F1 and are examining both male and female mice.

P27 deficient mice spontaneously develop pituitary adenomas, which might influence estrogen and progesterone levels via FSH and GH regulation through the ovaries, and hence indirectly affect breast tumor development. Thus, enhanced tumorigenesis in p27 deficient mice could be due to loss of p27 in the target mammary epithelial cells, or could be due to altered circulating hormone levels. To eliminate the latter possibility, we ovariectomized *Wnt-1* female mice of all three *p27* genotypes and implanted slow release pellets containing estrogen and progesterone. Thus all female mice regardless of *p27* genotype would have similar levels of these key hormones. This hormonal treatment was only done with the C57BL6 x 129 F1 but not the C57BL6 female mice. These experiments are ongoing, but the results to date indicate that p27 reduction in either male or female mice, either with or without hormonal treatment does not reduce the latency for mammary tumor development (Table 1).

Table 1: Median latency in weeks for breast tumor development in Wnt-1 transgenic mice:

p27 genotype	F1 male	F1 female	B6 male	B6 female
+/+	39.7 (n=4)	14.9 (n=22)	33.8 (n=6)	31.3 (n=22)
+/-	33.9 (n=10)	15.5 (n=27)	39.4 (n=10)	39.6 (n=23)
-/-	26.9 (n=2)	12.5 (n=17)	31.5 (n=3)	37.6 (n=6)

The number of male mice is lower, as most have not yet developed breast tumors. Note also the shorter latency for tumor development in the F1 females vs. the B6 females. This is due to the hormonal treatment given to the F1 females. Histologic analysis of the breast tumors indicates they are adenocarcinomas with no apparent difference between *p27* genotypes. There are two reasonable explanations for the apparent lack of effect of p27 reduction on mammary tumorigenesis using this model. One is that p27 does not act as a tumor suppressor in the murine breast, but does so in the human. There are well-documented differences in tumor spectra between humans and mice bearing germline mutations in tumor suppressor genes. Understanding the basis of this difference could be invaluable for interpreting future studies on mouse models of breast cancer. Second, it is possible that p27 does play a role in other murine models of breast cancer but does not cooperate with activated Wnt-driven tumorigenesis.

We have begun to address this by examination of cooperation of p27 with mutations in the tumor suppressor Apc, which is part of the Wnt signaling pathway. We crossed p27 deficient mice to *Apc* mutant *Min* mice. These mice spontaneously develop intestinal adenomas are also susceptible to ENU induced mammary tumor induction. The results clearly show that reduction of p27 greatly accelerates the latency and growth rate of intestinal adenomas driven by mutations in *Apc* (Figure 1). We next plan to treat these mice with ENU to induce breast tumors. If tumor latency is reduced in p27 deficient *Apc* mutant mice, this will demonstrate that p27 is a pathway specific breast tumor suppressor. It cooperates with mutations in *Apc* but not *Wnt*-1. If breast tumor latency is not accelerated in p27 deficient *Apc* mutant mice, this will demonstrate that tumor suppression by p27 is tissue specific. Understanding the basis for this will then be pursued.

Finally, as described in our last report, we have bred p27 -/- mice to p130 -/- mice through two generations to obtain p27-/- p130-/- compound mutant mice. Through 12 months of age the double mutant mice appear phenotypically normal and had not developed tumors. We have now followed these animals for an additional six months and have not observed any tumor development. Thus we have observed no synergy between p27-loss and p130-loss in tumorigenesis

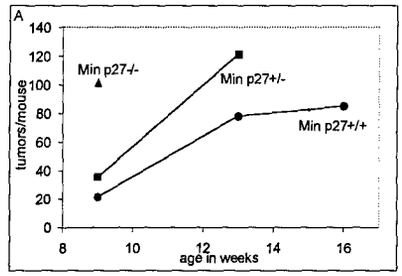

Figure 1. Reduction of p27 increases intestinal adenomagenesis in Min mice. Min p27-/- mice died at 9weeks due to extensive tumor burden. n=4-15 mice per group.

Task 3. Determination of p130 protein levels in primary human breast cancers, and determination of relative risk in patients with tumors expressing various levels of p130 and p27 proteins.

In this specific aim, we proposed to assess p130 and p27 expression in 600 paraffin-embedded primary breast tumor tissue samples collected as part of a population-based study of young women using immunocytochemical assays to determine the association between p130 and p27 expression in breast cancer and the association of p130 and/or p27 expression with relative risk of subsequent breast cancer or death.

We have analyzed the data from 119 cases using the Santa Cruz Biotechnologies, Santa Cruz, CA p130 antibody. We used a combination of staining intensity and percent cells with nuclear positive stain to establish high and low categories. The relationship of p130 expression and other patient and tumor characteristics is presented in Tables 2 and 3. In this small group of women, high p130 expression correlates with high proliferative rate (by Ki-67). Tumors with high p130 were also more likely to show overexpression of c-erbB-2, be PR negative and cyclin E positive but these associations did not reach statistical significance. As of the April 1999 follow-up of mortality, p130 was not associated with survival in this small group of women.

Table 2. Comparison of overall survival associated with combined p130/p27 expression

	Overall Survival		
	Alive	**Dead**	**HR***
P130/p27			
Hi/Hi	34	5 (12.8)	1.0
Lo/Hi	17	6 (26.1)	3.1 (0.9-11.4)
Hi/Lo	23	5 (17.9)	2.3 (0.6-8.7)
Lo/Lo	19	4 (17.4)	1.8 (0.5-7.2)

*HR adjusted for age, diagnosis year and stage

Table 3. Comparison of tumor characteristics to p130

Characteristic	P130		
	Negative/Low	Intermediate/High	P*
Nodal status			
Negative	27 (50.9)	33 (58.9)	
Positive	26 (49.1)	23 (41.1)	.402
Vital status – 5/99			
Alive	44 (80.0)	49 (84.5)	
Dead	11 (20.0)	9 (15.5)	.533
Stage			
Local	28 (50.9)	33 (56.9)	
Regional/Distant	27 (49.1)	25 (43.1)	.523
ER			
Positive	34 (61.8)	38 (65.5)	
Negative	21 (38.2)	20 (34.5)	.683
PR			
Positive	41 (74.6)	35 (60.3)	
Negative	14 (25.5)	23 (39.7)	.108

Ki-67			
Low	43 (78.2)	31 (53.5)	
High	12 (21.8)	27 (46.6)	.006
c-erbB-2			
Negative	35 (63.6)	27 (46.6)	
Positive	20 (36.4)	31 (53.5)	.068
p53			
Negative	39 (70.9)	35 (60.3)	
Positive	16 (29.1)	23 (39.7)	.238
BCL-2			
High	28 (50.9)	27 (46.6)	
Low	27 (49.1)	31 (53.5)	.643
Cyclin E			
Low	46 (83.6)	42 (72.4)	
High	9 (16.4)	16 (27.6)	.151
p27			
High	29 (52.7)	33 (56.9)	
Low	26 (47.3)	25 (43.1)	.656

*Pearson chi square

Key Research Accomplishments

- Establishment of murine model of p27-associated lymphomagenesis.
- Identification of several novel oncogenes that cooperate with p27-loss during lymphomagenesis.
- Establishment of methods for high-throughput analyses of retroviral insertion sites.
- Completion of pilot studies of DMBA administration to p27 -/- and +/- mice and observation of excessive death du to excessive death from pituitary tumors.
- Breeding of p27-null mice into p130-null, MMTV-wnt-1 transgenic, and Min strains and observation of affects on mammary epithelium/carcinogenesis.
- Optimization of p130 immunostaining in primary human breast cancer specimens.
- Initial characterization of the association between p130 and p27 expression in breast cancer and the association of p130 and/or p27 expression with relative risk of subsequent breast cancer or death.

A) Publications/manuscripts

1) Staheli-Philipp, J. Payne, SE, and Kemp, C.J. p27/Kip1: Regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. *Experimental Cell Res.* 264:148-168. 2001.

2) Jeannette Philipp-Staheli, Kyung-Hoon Kim, Shannon R. Payne, Kay E. Gurley, Denny Liggitt, Gary Longton, and Christopher J. Kemp*. Reduction of p27 synergizes with mutations in Apc to increase intestinal tumor growth and malignant progression. Manuscript in preparation.

3) Hwang, H., Martins, C., Berns, A., Fero, M., and B. Clurman. Identification of p27-cooperating oncogenes by insertional mutagenesis and high-throughput insertion site cloning, 2001. Manuscript in preparation

B) Funding Obtained

R01 CA84069-03 (P.I. B. Clurman) Mechanisms of p27-associted neoplasia.

C) Abstracts

1) Hwang., H, Randel, E., Fero, M., Roberts, J., and Clurman, B. 1999 Stage-Specific Gene Activations in P27-null Lymphomas. Abstract Presentation-American Society of Hematology Annual Meeting, New Orleans, La.

All Personnel Receiving Support from this Award

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Conclusions

The work completed in task 1 has validated the experimental strategy of using retroviral insertional mutagenesis to identify genes that cooperate with p27 loss in multi-step cancer. We have identified several novel and previously known oncogenes that cooperate with p27-loss in murine lymphomagenesis. We have also developed high throughput methods that take advantage of the sequenced mouse genome and informatics to greatly hasten cancer gene discovery by insertional mutagenesis. We are now determining the mechanism of synergy of these oncogenes with p27-loss, and examining their expression in human p27-associated cancers. This work may lead to important mechanistic insights into tumorigenesis associated with p27-loss.

The mouse models described in Task 2 were developed to examine the role of p27 in murine mammary carcinogenesis. The original goal of simply treating p27-null mice with carcinogens that induce breast tumors could not be achieved because of excessive

death due to pituitary tumors. Two additional strains were thus created to circumvent this problem, both of which involve components of the wnt pathway. As discussed above, thus far the data do not indicate that p27-loss synergizes with the wnt pathway in breast carcinogenesis.

Finally we used a mouse model and immunocytochemical analyses of human breast cancers to examine the interaction of the p130 protein with p27-loss. The basis for this work was previous observations that p130 functions as the principle inhibitor of cdk2 in cells lacking p27. However, neither the mouse nor human studies supported the hypothesis that p27 and p130 would cooperate in breast carcinogenesis.

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Appendix

p27^{Kip1}: Regulation and Function of a Haploinsufficient Tumor Suppressor and Its Misregulation in Cancer

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A major function of p27, also known as Kip1, is to bind and inhibit cyclin/cyclin-dependent kinase complexes, thereby blocking cell cycle progression. As p27 operates at the heart of the cell cycle, it is perhaps not surprising that it is emerging as a key player in multiple cell fate decisions including proliferation, differentiation, and cell death. The central role of p27 makes it important in a variety of disease processes that involve aberrations in cellular proliferation and other cell fates. Most notable among these processes is neoplasia. A large number of studies have reported that p27 expression is frequently downregulated in human tumors. In most tumor types, reduced p27 expression correlates with poor prognosis, making p27 a novel and powerful prognostic marker. In addition to these practical implications, murine and tissue culture models have shown that p27 is a potent tumor suppressor gene for multiple epithelially derived neoplasias. Loss of p27 cooperates with mutations in several oncogenes and tumor suppressor genes to facilitate tumor growth, indicating that p27 may be a "nodal point" for tumor suppression. In contrast to most tumor suppressor genes studied to date, which are recessive at the cellular level, p27 is haploinsufficient for tumor suppression. The fact that tumor suppression by p27 is critically dependent on the absolute level of p27 expression indicates that p27 acts as a rheostat rather than as an on/off switch to control growth and neoplasia.

INTRODUCTION

p27 was initially identified in complexes with cyclin E/cyclin-dependent kinase (CDK)2 in cells that had been growth arrested with TGF-β, contact inhibition, or lovastatin [1, 2]. It had been known for several years prior to this discovery that cyclin E activated CDK2 and that CDK activation was a key event in driving cells from G1 into S phase. Interestingly, the activity of CDK2 was inhibited by bound p27 indicating that p27 was a cyclin-dependent kinase inhibitor (CDKI). The regulation of cyclin E/CDK2 by p27 generated intense interest, as TGF-β had been recognized for many years as a potent physiological inhibitor of cell proliferation, although its mechanism of growth inhibition remained largely undefined. Subsequent studies demonstrated that other physiological agents that influenced cell proliferation such as contact inhibition, cAMP, rapamycin, and IFN-γ also acted through p27 [reviewed in 3–5). As activation of CDKs is at the core of cell cycle progression, p27 inhibition of CDKs suggested a direct connection between extracellular signals and cell cycle progression. The mechanistic basis for p27 activity has since emerged from detailed biochemical analysis and from the analysis of the crystal structure of p27 bound to cyclin–CDK complexes [6]. Since those early studies focusing on proliferation, hundreds of reports using a variety of agents and cell systems have investigated the role of p27 in a much wider range of cellular processes, such as differentiation, apoptosis, and migration. As these processes are frequently misregulated in disease states, more recent work has focused on the role of p27 in disease progression. Most relevant to this review, p27 has been implicated as a critical player in the progression of a surprisingly wide variety of human neoplasms. We will focus on the many functional properties attributed to p27 with specific attention to the similarity between p27 haploinsufficiency observed in mouse models of tumorigenesis and the expression level of p27 in human tumors.

p27 GENE STRUCTURE

The human *p27^{kip1}* (hereafter *p27*) gene resides in a region of chromosome 12p13 that is frequently rearranged or deleted in hematologic malignancies [7–10]. It contains two coding and one noncoding exons. The mouse *p27* gene spans 3.6 kb and is located in a syntenic region on distal chromosome 6 [11]. The exon–intron structure of the mouse *p27* gene is similar to the human *p27* gene and its cDNA sequence is more than 90% homologous to the human p27 cDNA.

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p27 MUTATION IN HUMAN CANCER

CDKIs such as p16 and p15 are frequently inactivated both in the germline and somatically in a number of human cancers. When *p27* was cloned, scientists searched for *p27* genetic alterations in human malignancies as well. LOH within the *p27* region as defined by the markers D12S133 proximally and D12S142 distally is a common feature of some human cancers, including acute myeloid leukemia, acute lymphoblastic leukemia, ovarian cancer, and prostate cancer [9, 12–14].

Of the more than 500 tumors analyzed, however, only a handful of somatic mutations in *p27* have been detected [see for example, 15). Although the majority of bone marrow samples from acute myeloid and acute lymphoblastic leukemia patients show hemizygous deletion of *p27* by fluorescent *in situ* hybridization (FISH), no inactivating mutations in the remaining *p27* allele have been observed [9]. Analysis of 66 breast tumors revealed only one inactivating mutation of *p27,* a point mutation leading to the creation of a nonsense codon at position 104 and premature protein truncation [16, 17]. This somatic mutation was accompanied by LOH of markers near *p27* on chromosome 12p13, indicating that loss of p27 played an important role in the genesis of the invasive ductal carcinoma. Additionally, homozygous deletion of the *p27* gene was detected by Southern hybridization in one B-immunoblastic lymphoma and in one acute adult T-cell leukemia/lymphoma, indicating that *p27* genetic loss plays a role in the development of at least a small percentage of hematologic malignancies [18].

Importantly, these studies relied on a combination of PCR-based mutation analysis (e.g., SSCA and sequencing) and Southern hybridization to detect the low frequency of *p27* mutations in human cancers. Thus, it is evident that mutations of *p27,* either by insertion/deletion/point mutation or by large rearrangement, occur very infrequently in all human cancers examined to date.

ALTERNATIVE METHODS OF p27 INACTIVATION

Unlike classic tumor suppressor genes such as *TP53* and *RB1,* the *p27* gene contains only rare somatic mutations and shows a low frequency of allelic loss. Several explanations for the absence of *p27* gene mutations in human cancer can be considered. One formal explanation would state that p27 is not targeted in human cancer and therefore cannot be considered a tumor suppressor gene. As described in detail below, this is almost certainly not the case given the large body of data indicating the prognostic significance of p27 protein expression in a variety of epithelially derived cancers [reviewed in 5, 19].

A second explanation would be the existence of an alternative mechanism for the inactivation of the second *p27* allele in tumors with allelic loss. Aberrant patterns of DNA methylation are among the most common genomic alterations seen in cancer [reviewed in 20]. Few detailed studies of *p27* methylation status in normal and transformed human tissue have been published. In mouse and rat pituitary cell lines, p27 expression has been shown to be regulated by DNA methylation [21]. However, human pituitary adenomas do not exhibit LOH for the *p27* region [22], still necessitating an explanation for inactivation of the remaining *p27* allele.

A third possibility is that p27 is inactivated at a posttranscriptional level. Comparison of immunohistochemical staining and *in situ* hybridization reveals discordance between p27 mRNA and protein levels in human breast, colon, and pituitary tumors [23–25]. Recently, an element within the 5′-untranslated region (UTR) of the p27 mRNA has been described that is required for efficient translation of p27 in proliferating and quiescent cells [26]. The proteins that bind *in vitro* to this p27 5′ UTR element, HuR and hnRNPC1, are governed in a cell-cycle-dependent manner. Thus, translation of p27 mRNA may depend on specific factors that are regulated by the cell cycle. The mutation status of genes whose proteins bind to the p27 5′ UTR and posttranslational modifications of such proteins warrant further investigation in human cancers.

By far, the most common methods of inactivating p27 occur at the posttranslational level. Altered subcellular localization of p27 and degradation of p27 by the ubiquitin–proteasome pathway are observed in a number of human cancers. Aberrant cytoplasmic localization of p27 is more common in colorectal, ovarian, and Barrett's esophagus-associated adenocarcinomas than in adjacent normal tissue where p27 exhibits moderately intense nuclear staining [24, 27–30]. *In vitro* studies indicate that cytoplasmic localization of p27 contributes to anchorage-independent growth [31]. However, Barrett's esophagus-associated adenocarcinoma is the only cancer in which cytoplasmic localization of p27 is significantly associated with relevant prognostic indicators [28]. Aberrant regulation of p27 protein stability is a clinically important mechanism for inactivation of p27 in human glioma, colon, non-small cell lung cancer, and mantle cell lymphoma [24, 32–35]. In these tumor types, reduced p27 protein is directly correlated with increased proteasome-dependent degradation of p27.

In summary, while loss of one *p27* allele is frequent in many human cancers, mutation of the remaining allele is rare. This situation is strikingly similar to that seen in *p27*+/− mice in which *p27* is haploinsufficient for tumor suppression [36].

REGULATION OF p27

In many cellular systems, p27 is regulated posttranscriptionally, at the level of both protein translation and protein stability. While multiple mechanisms have been proposed for the regulation of p27 gene expression, current data support a major role for the ubiquitin/proteasome pathway in regulating p27 abundance. *In vitro* and *in vivo* findings show that active cyclin/CDK2 complexes are required for the elimination of p27. Cyclin E/CDK2 is the main target that is inhibited by p27. Reciprocally, cyclin E/CDK2 phosphorylates p27 at Thr187, thereby triggering its ubiquitination and degradation by the proteasome complex. p27 binds to cyclin E/CDK2 with high and low affinities [37]. Initially, p27 binds with low affinity acting as a substrate, and then slowly the binding shifts to high affinity and p27 becomes an inhibitor [37]. At equilibrium, p27 inhibits cyclin E/CDK2 activity. This mechanism provides a negative regulatory feedback loop that makes G1/S transition irreversible [37–39].

Molecules must be ubiquitinated in order to be recognized by the proteasome complex for degradation. Two proteins are known to ensure correct timing of p27 elimination. p27 is phosphorylated on threonine residue 187 by CDK2 and possibly other kinases and this targets p27 for ubiquitination. Recently, the ubiquitin protein ligase for p27 has been identified as $p45^{SKP2}$ (SKP2) [40, 41]. SKP2 is the substrate targeting subunit of the ubiquitin–protein ligase SCF^{SKP2} and is frequently overexpressed in transformed cells. Ectopic expression of SKP2 in quiescent cells leads to cytoplasmic accumulation of p27, promotes p27 degradation, and induces S phase in a phosphorylation-dependent manner [40, 41]. A mutant p27 protein (T187A), which cannot be phosphorylated at T187, is resistant to CDK2, SKP2, and proteasome-mediated degradation [40]. Thus, both CDK2 and SKP2 are rate limiting for p27 degradation and the transition from quiescence into a proliferative state. p27 ubiquitination in cell extracts depends on the presence of the ubiquitin-like protein Nedd8 and enzymes that catalyze Nedd8 conjugation to proteins [42]. Modification of cullin-1, a component of SCF, by Nedd8 enhances the activity of SKP2 toward p27 [43]. In normal cells, SKP2 expression is restricted to S phase [44, 45]. Thus, its constitutive expression in transformed cells may contribute to reduction of p27 and deregulated DNA replication in cancer. In fact, targeted disruption of the *Skp2* gene in mice results in accumulation of cyclin E and p27 as well as polyploidy and centrosome overduplication, but not cancer [46]. This result suggests that specific degradation of p27 and cyclin E is mediated by the SKP2–ubiquitin–ligase complex and controls the abundance of these cell cycle regulators *in vivo*. It is not clear, however, whether the accumulation of p27 is essential for the polyploidy and centrosome overduplication observed in the *SKP2*-deficient mice. In fact, a study using an adenovirus vector system to overexpress p27 in human brain tumor cells discovered a marked decrease in the accumulation of aneuploid cells and diminished malignant potential [47].

Different kinase signal transduction pathways have been shown to result in phosphorylation of p27 leading to its degradation. Oncogenic Ras confers anchorage independence by accelerating p27 degradation through activation of the MAP kinase signaling pathway [48]. In fact, *in vitro* assays have confirmed that MAP kinase is able to phosphorylate p27 in a cell-free system [48]. However, several groups have shown that ras signaling induced by growth factors (PDGF, EGF, serum) or integrins leads to increased cyclin D expression via the ERK signaling pathway and to p27 degradation due to phosphorylation by activated cyclin E/CDK2 [49–53]. The protein responsible for p27 degradation by means of regulation of cyclin E/CDK2 is the small GTP-binding protein RhoA [52]. Thus, ras induces G1 progression and p27 inactivation through stimulation of two independent pathways: stimulation of the ERK pathway leads to an increase in cyclin D protein which can sequester p27; stimulation of RhoA is necessary for p27 degradation by the proteasome pathway. Activation of Rho proteins through geranylation is essential for the degradation of p27 in a growth-stimulated rat thyroid cell line [54]. However, it is unclear whether active RhoA acts directly or indirectly on p27 to target it for ubiquitin-mediated degradation. Activation of cyclin E/CDK2 upon growth factor stimulation also ensures transcriptional activation of the cyclin A promoter and an increase in cyclin A expression, an event that is blocked by p27 [55]. Other kinases implicated in p27 degradation include PI3 kinase (PI3K) and the key cell survival kinase AKT, a direct target for PI3K phosphorylation. PI3K activity is required for the degradation of p27 following CD40 induced proliferation of activated B-cells, independent of AKT [56]. AKT activity is markedly increased in a human prostate cancer cell line and constitutively active AKT leads to direct inhibition of AFX/Forkhead-mediated transcription of p27 and to increased cyclin D [57]. Although ras activation has a major impact on p27 regulation, the specific kinase pathway leading to downregulation of the p27 protein depends on the cell type and stimulus.

Although the importance of Thr187 phosphorylation in ubiquitin-mediated degradation of p27 is undisputed, it was recently discovered that phosphorylation of Ser10 accounts for 70% of the total phosphorylation of p27 and is regulated in a cell-cycle-dependent manner [58]. In contrast to Thr187, phosphorylation of Ser10 increases the protein stability of p27. However, both the kinase and the phosphatase responsible for

phosphorylation and dephosphorylation of Ser10, respectively, as well as the mechanism by which phosphorylation of Ser10 stabilizes p27, remain to be identified. It will be interesting to see if regulation of p27 stability by phosphorylation of Ser10 is linked to external mitogenic signals.

While p27 levels are generally constant during the cell cycle, there are certain circumstances when p27 is regulated at the transcriptional level. Normal human quiescent T-cells express high levels of p27 mRNA and protein, which rapidly decline within 5 h after polyclonal activation [59]. Regulation at the mRNA level also occurs after androgen deprivation of mammary carcinoma cells [60], in normal prostate tissue, and in benign prostatic hyperplasia [61]. Some possible transcriptional regulators of p27 have recently been described. The promoter region of the p27 gene contains binding sites for several transcription factors including Sp1, CRE, Myb, NFκB, and AFX. AFX is a member of the forkhead family of transcription factors and has recently been shown to induce a p27-mediated cell cycle arrest in a variety of cell lines, including a Ras-transformed cell line and a cell line lacking the tumor suppressor PTEN [62]. AFX integrates the PI3K/PKB and Ras/Ral signaling pathways to regulate transcription of p27. In prostate cancer, inactivation of the tumor suppressor gene PTEN leads to constitutive activation of AKT via the PI3K pathway. AKT phosphorylates and inactivates the forkhead transcription factor AFX and suppresses p27 gene transcription [57]. STAT3 is an IL-6 inducible transcription factor, which is relocated from the cytoplasm to the nucleus after appropriate stimulation. In an IL-6-sensitive melanoma cell line, signaling through the IL-6 receptor leads to STAT3 activation and an increase in p27 mRNA [63]. Enhanced ribosomal association of p27 mRNA is yet another mechanism that contributes to accumulation of p27 during growth arrest in TPA-treated HL-60 cells [64].

SEQUESTRATION

The sequestration of p27 and also p21 into higher-order complexes with cyclin D serves two different purposes. p27 promotes the assembly of cyclin D/CDK4 complexes by binding to them [65–68] making the cyclin D/CDK4-bound p27 unavailable for cyclin E/CDK2 binding. The unbound cyclin E/CDK2 can become activated and drive the cell through G1/S phase. Cheng and colleagues discovered that the MEK/ERK pathway not only acts transcriptionally to induce the cyclin D1 gene but functions posttranslationally to regulate cyclin D1 assembly with CDK4, thereby preventing p27-mediated inhibition [69].

The protooncogene c-*myc* is involved in the regulation of cyclin E/CDK2 activity [70, 71] and plays a major role in p27 sequestration through modulation of cyclin D and E protein levels [72–74]. c-myc may induce additional p27-sequestering proteins [75, 76] that are as yet unknown. The relative contribution of each of these p27-binding proteins to cell cycle control in normal cells and in cancer cells may vary with tissue context. c-myc can be induced by several pathways. Activation of the MAP (Erk1/2) kinase pathway leads to stabilization of the c-myc protein [77] and increased cyclin D transcription [78]. Furthermore, activation of the PI3K/PKB pathway has been implicated in the translational induction of c-myc [79] and stabilization of D-type cyclins [68, 80]. The fact that both ras and c-myc are impinging on p27 regulation explains in part why coexpression of Ras with c-myc allows the generation of cyclin E/CDK2 activity and entry into S phase, coincident with the loss or sequestration of the p27 protein [71].

CYTOPLASMIC DISLOCATION

It has become increasingly clear that subcellular localization of p27 plays a major role in its regulation. In order to exert its function during G1 and to be degraded at the end of G1, p27 needs to be imported into the nucleus [81, 82]. This may be due to the fact that the localization of CAK, which is an essential activator for cyclin E/CDK2, is restricted to the nucleus [83]. The C-terminal region of p27 contains a nuclear-localization signal (amino acid residues 153–170), guiding p27 into the nucleus in normal cells. Recent findings indicate that the nuclear-pore-associated protein mNPAP60 is required for intracellular transport and, hence, cyclin E-mediated elimination of p27 [84]. The interaction of p27 with mNPAP60 was discovered in a two-hybrid screen and the interaction mapped to the 3_{10} helix of p27. A point mutation in p27 that abolishes its interaction with mNPAP60 leads to poor nuclear import of p27, accumulation of phosphorylated p27 in the nucleus, and impaired degradation. Cells containing this mutated form of p27 also show enhanced inhibition of proliferation. Since proteasomes accumulate at the nuclear periphery [85, 86], interaction of mNPAP60 with p27 may ensure efficient presentation of the ubiquitinated p27 to the degradation machinery.

One of the key proteins for nuclear export of p27 was recently shown to be JAB1. JAB1 was originally identified as a component of the 450K COP9/signalosome complex [87–89] which phosphorylates IκB, the NF-κB precursor and c-Jun [87] and is structurally similar to the proteasome regulatory complex [90]. The JAB1 protein causes the translocation of p27 from the nucleus to the cytoplasm, leading to increased p27 degradation. Binding to JAB1 and nuclear export is required for degradation and therefore p27 activity might be regulated at the transport step [82]. Whereas binding of

JAB1 to p27 occurs independent of phosphorylation, a phosphorylation step is needed for proteasome-dependent degradation. Whether JAB1 and mNPAP60 act sequentially in a single pathway or in parallel pathways directing export of p27 from the nucleus will be an interesting point of further investigation. A role for JAB1 and p27 in the inhibition of interleukin 2 (IL-2) production has emerged from studies on helper T-cells. p27 is an essential regulator responsible for the blockade of clonal expansion of anergic T cells *in vitro* and *in vivo* [91]. In anergic cells, p27 associates with JAB1, resulting in defective transactivation of AP-1 and IL-2 transcription.

Findings *in vivo* indicate that improper subcellular regulation of p27 may lead to deregulation of the cell cycle and a transformed phenotype. In transformed and tumor cell lines which are anchorage independent more than half of the p27 protein can be found in the cytoplasm where it is bound to cytoplasmic cyclin E/CDK2, cyclin A, and cyclin D [31]. Cytoplasmic displacement of p27 has been observed in tumors of the colon, esophagus, and thyroid gland [27, 28, 92]. In thyroid tumors, cytoplasmic localization of p27 is found in 80% of the cases and is associated with cyclin D3 overexpression [92]. In these cases, a cytoplasmic p27–cyclin D3/CDK complex is formed and sequesters p27 away from complexes containing cyclin A–E and CDK2. D-type cyclins lack a nuclear-localization domain that allows their entry into the nucleus, but their nuclear translocation may occur after they bind to CDKIs or CDKs [37, 66]. On the other hand, p27 is relatively inefficient in targeting cyclin D/CDK complexes to the nucleus [37, 66, 81] and cytoplasmic retention of cyclin D3/p27 complexes could be due to the presence of a specific cytoplasmic signal in cyclin D3 that is dominant over p27's nuclear-localization signal. It seems that tumor cells ensure cell cycle progression by a number of different measures. Orend and colleagues discovered that transformed fibroblasts and tumor cells, which are anchorage independent, have more than half of their cyclin E, CDK2, p21, and p27 in the cytoplasmic fraction, both in attached and in suspended cells [31]. Three factors allowed unhindered cyclinE/CDK2 activation in transformed cells: upregulation of cyclin E and CDK2, sequestration of p27 and p21 away from the nucleus by cytoplasmic cyclin/CDK complexes (complexes containing cyclin E as well as cyclin A and cyclin D), and impaired binding of p27 and p21 to nuclear cyclin E/CDK2 complexes.

Other proteins involved in cancer lead to cytoplasmic displacement of p27. Loss of the tuberous sclerosis complex gene-2 (*TSC2*) causes mislocalization of p27 into the cytoplasm concomitant with decreased protein stability [93]. Tuberous sclerosis is an autosomal dominant disorder characterized by the development of aberrant growth in many tissues and organs. *TSC2*-negative fibroblasts exhibit a shortened G1 phase, accompanied by elevated CDK2 activity, most likely due to the absence of sufficient p27 protein. Overexpression of HER-2/*neu* plays a pivotal role in the formation of a number of human cancers and causes enhanced ubiquitin-mediated degradation of p27 in breast cancer cell lines [94]. The HER-2/Grb2/MAPK pathway is involved in decreasing p27 stability and leads to nuclear export of p27 and JAB1 and subsequent p27 degradation.

Most of the above described studies were performed in cell culture, and the regulation of p27 expression *in vivo* in normal tissue or in tumors is less understood. Rare studies of p27 regulation in human tumors suggest that low p27 results from enhanced degradation as seen in colon tumors [24] or from the exclusion of p27 from the nucleus as in Barrett's associated adenocarcinoma [28]. Thus there may be several ways to misregulate p27 expression during tumorigenesis *in vivo*.

CELLULAR BIOLOGY OF p27

The mediation of mitotic and antimitotic extracellular signals has emerged as a key feature of p27 making it distinct from other members of the Cip/Kip family. Generally, p27 functions as a major gatekeeper of the quiescent state in mammalian cells [3]. p27 protein is abundant in quiescent cells, in contact-inhibited cells, and in cells treated with antimitotic stimuli. In contrast, p27 expression is low in certain aggressively growing tumors and after mitotic stimulation. Several cellular and viral oncogenes are able to downregulate p27 protein. However, levels of p27 protein are also found to be normal even in certain aggressively growing tumors and in transformed cell lines until growth conditions are changed by serum withdrawal or mitogen stimulation. Recently, a variety of new functions for p27 cellular have been discovered, including roles in apoptosis, cell–cell adhesion, drug sensitivity, differentiation, and tumor virus biology. Thus, it seems that the regulation and role of p27 in different cellular settings is quite complex and its detailed study will add crucial knowledge to our understanding of tumor development.

REGULATION BY PHYSIOLOGICAL SIGNALS

p27 is the only CDKI known to date that has a unique role as a mediator between cell cycle and mitotic stimuli. Various mechanisms of inducing growth arrest in normal cultured cells increase p27 levels including TGF-β [95, 96], lack of IL-2 [97], low serum [98], cellular confluence [97], and growth in suspension [99]. Some of these physiological signals are deregulated in cancer, with a direct effect on p27 levels and

the cell cycle. For example, TGF-β inhibits the growth of normal epithelial cells and relatively differentiated carcinoma cells, whereas dedifferentiated tumor cells, lacking many epithelial characteristics, are usually resistant to growth inhibition by TGF-β [100]. In addition, collaboration with ras increases the invasive potential of epithelial tumor cells [101]. In TGF-β-arrested epithelial cells, p27 is competing with $p15^{INK4B}$ for binding to cyclin D1/CDK4 [97, 102, 103]. Upregulation and binding of p15 to CDK4 serves to destabilize the association of p27 with cyclin D1/CDK4 and promotes p27 binding to cyclin E/CDK. Indeed, in TGF-β-induced epithelial cells, upregulation of p15 protein and increased binding of p15 to cyclin D1/CDK4 occurs concomitant with reduction of CDK4-associated p27 and stabilization of the association of p27 with cyclin E/CDK2 complexes [103]. However, Florenes *et al.* [104] point out that this is not a universal mechanism of TGF-β-induced G1 arrest. In an early-stage human melanoma line, which lacks p15, both p21 and p27 contribute to G1 arrest induced by TGF-β, emphasizing the importance of cell-type-specific variations in the effects of TGF-β on cell cycle regulators.

p27 mediates responses to several other growth inhibitory signals, including cAMP in macrophages [105], rapamycin in T-lymphocytes [97], IFN-γ in mammary epithelial cells [106], interferon-β in human gastric cells [107], and IL-6 in melanoma cells [63]. Interferons also induce p27 and p21 in a human Burkitt's lymphoma and an acute promyelocytic cell line, preventing activation of CDK-activating kinase (CAK) [108]. Signaling through the receptors for oncostatin M (OSM) and IL-6 leads to accumulation of p27 and G1 arrest and seems to involve the extracellular signal regulated kinase (ERK) and STAT5 pathways [109]. OSM and IL-6 both inhibit growth of melanoma and lung cancer cells [110, 111]. Although cyclin E/CDK2 and other CDK2-containing cyclins seem to be the principal targets in p27-mediated growth arrest, vitamin D-treated promyelocytic leukemia cells show a specific increase in CDK6-associated p27 protein [112]. p27 has also been demonstrated to mediate G1 arrest mediated by a number of drugs, including rapamycin and lovastatin, a potent inhibitor of ras [1, 97].

On the other hand, several growth factors trigger a rapid decrease in p27 expression. Estrogens stimulate cell cycle progression in breast cancer cells through loss of p27 and p21 [113]. In T-cells, IL-2 causes a decrease in p27, allowing CDK2 activation and entry into S phase [97]. Stimulation with mitogens like PDGF or serum also leads to p27 downregulation and growth arrest in 3T3 fibroblasts [114], in quiescent fibroblasts [98], and epithelial cells [115]. Upon downregulation of p27, cells progress through the G1 phase and often become independent of mitogens. Cancer cells have various means of deregulating p27 expression and to render it unable to interfere with cyclinE/CDK2 induction. Cells that are transformed by polyamines due to upregulation of ornithine decarboxylase, a situation commonly seen in cancer cells, display constitutive downregulation of p27 and its loss from the cyclinE/CDK2 complexes [116].

In summary, a variety of physiological proteins impact p27 function in different ways depending on tissue and cellular differentiation state. These different modulators of cellular fate are likely to regulate p27 expression by multiple and distinct mechanisms in normal and neoplastic tissues.

VIRAL INHIBITORS

A key feature of many DNA tumor viruses is their ability to interfere with the normal cell cycle of their target cells. Several viral gene products have been shown to bind and inactivate important components of the cell cycle machinery. Some viral oncoproteins interact directly with pRb thereby bypassing the need for cyclin/CDK activity to release E2F and allow the cells to undergo G1/S transition. Other viral targets include p53 and its downstream mediator p21. Thus, the guardian function of p53 during DNA-damaging events can be circumvented as well. Recent evidence shows that p27 can also be a target for DNA tumor viruses and that these viruses use different mechanisms to prevent growth arrest by p27.

The adenovirus oncoprotein E1A can not only bind directly to pRb to overcome TGF-β-induced cell cycle arrest, but also directly affects p27 by binding to it and blocking its inhibitory activity in mink lung epithelial cells [117]. This view has been challenged recently by a report by Alevizopoulos *et al.* showing that free p27 levels in E1A-expressing Rat1 cells are increased, and yet the cells are proliferating [118]. The authors postulate that E1A targets pRb family members and other yet unknown effectors of CDK2 in G1/S control. The finding that E1A overcomes p27-induced growth arrest without releasing an inhibition of CDK2 activity is in sharp contrast with the effect of c-myc, which prevents CDK2 inhibition by inducing sequestration of p27 within cells into a form unable to associate with CDK2 [75, 76]. Thus, c-myc overcomes p27-induced arrest by a mechanism sensitive to p27 overexpression, while E1A allows cell growth with supraphysiological p27 levels and maximally inhibited CDK2.

The E7 oncogene of HPV-16 induces S phase entry of mammalian cells in the presence of antiproliferative signals. Binding of E7 to and subsequent inactivation of p27 is in part responsible for the virus' ability to override certain forms of G0/G1 arrest [119]. The polyoma virus small T antigen (Py sT) triggers phosphorylation and degradation of p27 protein indirectly by

binding to protein phosphatase 2A (PP2A) [120]. The interaction between PP2A and Py sT has been shown to induce the MAPK pathway, which could be one mechanism for phosphorylation of p27. Other viral gene products that inhibit p27 activity and induce growth-factor-independent growth include the v-ras oncogene and v-src. Ras activation can lead to proteasome-dependent degradation of p27 through phosphorylation via the MAPK or PI3K pathway [48, 56]. The v-src oncogene decreases p27 protein by interfering with its transcription and therefore downregulates p27 mRNA [121]. Interestingly, the Epstein–Barr virus ensures its lytic replication by inducing G1 arrest in cells. The EBV-encoded immediate-early transcription factor, Zta, can induce expression of cyclin-dependent kinase inhibitors, p27 and p21, as well as p53, independent of its transcriptional transactivation function [122].

APOPTOSIS

Apoptosis in tumors has become a subject of considerable interest in recent years. A balance among proliferation, growth arrest, and apoptosis regulates cell numbers. In certain tumors, an association between patient survival, clinical outcome, and the extent of apoptosis has been demonstrated in clinical studies. Growing evidence suggests that apoptosis frequently occurs in cells in the G1 phase of the cell cycle [123, 124], and arrest in late G1 or S phase can accelerate or potentiate apoptosis [125]. Thus, proteins expressed during late G1 like p21 and p27 are logical targets for the apoptotic process.

The first notion that p27 may play a role in apoptosis came from experiments using adenoviral vectors to overexpress p27 in cancer cell lines. p27 induced apoptosis in these studies whereas additional research, mostly in normal cells and *in vivo,* pointed toward a protective role in apoptosis for p27.

Recombinant adenovirus-overexpressing p27 induces not only cell cycle arrest and loss of cyclin–CDK activity but also triggers apoptosis in human breast cancer cells and several other cancer cell lines [126, 127]. Adenoviral expression of the closely related p21 in the same cells only leads to G1 arrest and minimal apoptosis. Naruse *et al.* found that p27-induced apoptosis in lung cancer cell lines required the presence of pRb [128]. In this study, the maximal apoptotic response due to adenoviral p27 expression occurred at 72 h after infection and apoptosis was not induced in the total cell population. Thus, it is possible that cells arrested in G1 may be protected from apoptosis. Indeed, p21 has been postulated to have an antiapoptotic effect, presumably by causing G1 arrest [129]. Apoptosis induced by a proteasome inhibitor in several solid tumors cell lines, correlated with p27 protein expression, again pointing to a proapoptotic role for p27 [130]. In these studies, the effect of p27 on apoptosis was clearly different in normal versus tumor cells. Induction of apoptosis by proteasome inhibitors and concomitant p27 induction only occurred in SV40 transformed fibroblasts but not in the parental fibroblasts. *In vivo,* spontaneous apoptosis in some human cancers with high p27 expression is significantly higher than in tumors with low p27 levels and has been shown to correlate with BAX expression in oral and oropharyngeal carcinomas [131]. The combined effect of p27 on G1 arrest and apoptosis in various cancer cell lines makes adenoviral vectors containing p27 an interesting tool for gene therapy. Indeed, adenoviral vectors containing both p27 and p16 have already shown some promise in mice and may represent a powerful new therapeutic agent for cancer gene therapy [132].

In contrast to the studies discussed above, others have reported antiapoptotic effects of p27. In carcinoma cells and in leukemic cell lines p27 has been shown to prevent drug-induced apoptosis [133–135]. Normal fibroblasts and mesangial cells from p27-deficient mice undergo increased apoptosis after cyclin A/CDK2 activation following growth factor deprivation [136]. Exogenous expression of p27 in these cells inhibits apoptosis induced by serum withdrawal. Levkau and colleagues show that p27 and p21 undergo specific cleavage by CPP32 and/or a CPP32-like caspases upon serum-deprivation of endothelial cells [137]. Cleavage of these two proteins results in reduction of their association with CDK2 and a drastic activation of cyclinA/CDK2. Inhibition of p21 cleavage as well as inhibition of cyclin A/CDK2 activity protects cells against apoptosis. This suggests that cleavage of p27 and p21 results in activation of cyclin A/CDK2 and that this is a necessary step leading to apoptosis in this epithelial cell line.

Apoptosis in cells of the hematopoietic system involves similar pathways. Stable transfection of leukemic cells with p27 results in inhibition of apoptosis induced by various stimuli including DNA-damaging agents and anti-Fas antibodies [135]. Eynim *et al.* show that the protection from apoptosis is due to the generation of p23 and p15 N-terminal cleavage products of p27 by caspases, mainly caspase 3 [138]. These cleavage products prevent procaspase-3 activation in etoposide-treated cells and represent a protective negative feedback loop. As opposed to p21, apoptosis-related p27 cleavage does not correlate with increased CDK2 activity nor with relocalization of p27 to the cytosol. Thus, p27 fragments may act upstream of the activation of distal caspases by preventing cytochrome *c* release from the mitochondria [139]. Whatever the mechanism of action, p27 is the first cellular protein whose caspase-mediated cleavage is shown to generate peptides that negatively regulate the death process,

providing an additional mechanism for the cell to control apoptotic pathways.

Whether p27 promotes or inhibits apoptosis depends in part on the cleavage status of p27, cell type, and the status of the cell, transformed versus nontransformed. Cancer cell lines, by their nature, are aberrant in cell cycle regulation, and it is therefore not surprising that associations discovered there are different from findings in normal diploid cells and tissue. It may be that certain tumor cells have a mechanism whereby they induce cleavage of p27, which could lead to decreased apoptosis. Such a mechanism would be one explanation why low p27 expression often correlates with poor survival for patients.

DIFFERENTIATION

A function for p27 during differentiation has been inferred mainly from its increased expression in a variety of terminally differentiated cells [140, 141]. It was thought at first that p27 would only be responsible for the G1 arrest preceding cellular differentiation programs. It is now clear that p27's role in differentiation is more sophisticated. Constitutive expression of p27 in NT2/D1 cells, for example, not only induces a marked reduction in the growth potential of these cells but also triggers the appearance of some differentiation markers [142]. Likewise, overexpression of p27 in a colon cancer cell line leads to partial growth inhibition and to an increase in sensitivity to induction of differentiation [143]. Growth arrest alone has been proven in several systems to be insufficient for differentiation in a variety of cell types. Dissection of p27 function during differentiation has allowed the uncoupling of growth arrest from true differentiation events.

The role of p27 as an important factor in differentiation has also been established in various physiological settings using cell lines. Granulocyte colony-stimulating factor (G-CSF) regulates granulocytic differentiation by inducing a robust and sustained activation of the transcriptional activator STAT3 [144]. p27 mRNA expression is induced by G-CSF and the p27 promoter has a functional STAT3-binding site [145]. The fact that myeloid progenitor cells from p27-deficient mice show increased proliferation and reduced differentiation in response to G-SCF indicates that STAT3 controls myeloid differentiation, at least partly, via upregulation of p27 [145]. GM-CSF and all-*trans*-retinoic acid synergistically induce granulocytic differentiation in human myeloblastic leukemia cells, concomitant with an increase in CDK2-bound p27 [146, 147]. Retinoic acid alone has the same effect without affecting p27 expression and was shown previously to increase p27 protein and p27 binding to CDK2 in differentiating neuroblastoma cells [148]. Retinoic acid can lead to degradation of D-type cyclins, which may allow release of p27 from cyclin D complexes and allow its transfer to CDK2-containing complexes [149, 150]. Terminal differentiation of erythroid cells results in cell divisions followed by irreversible cell cycle withdrawal of hemoglobinized cells. During erythropoietin-induced terminal differentiation of erythroblasts, p27 binding to cyclin E- and cyclin A-associated CDK2 correlated with the inhibition of these kinases [151–153]. Binding of p27 to CDK4 and to a lesser extent CDK6 early during differentiation has no apparent effect on these kinase activities. Although accumulation of p27 during erythrocyte differentiation is associated with growth arrest, overexpression of p27 does not promote terminal erythroid differentiation.

p27 has also been implicated in keratinocyte and intestinal epithelial cell differentiation. While p27 accumulates in keratinocytes during differentiation, pretreatment of adherent cells with p27 antisense oligonucleotides prevents the onset of differentiation [141]. Increased p27 expression in human intestinal epithelial cells during differentiation is delayed as compared to p21 and p27 fails to complex in significant amounts with any of the G1 cyclins. This led to the suggestion that p27 may induce or stabilize expression of differentiated traits that act independently of cyclin–CDK function, while p21 is required for the G1 arrest [154]. Hauser *et al.* suggest that a novel pRb kinase activity present in p27 immune complexes, which is dependent on the presence of cyclin D3, would represent such a cyclin/CDK-independent function [141]. Indeed, adenoviral expression of p27 is followed by the induction of differentiation markers in human intestinal epithelial cells at the mRNA and protein level [155]. Contrary to this observation, forced expression of p27 in normal human epidermal keratinocytes is insufficient to induce squamous differentiation [140]. Additional signals generated during suspension culture are required to promote the complete differentiation program. It is possible that this reflects differences in the intrinsic properties of these two epithelia. *In vivo,* epidermal keratinocytes form a stratified epithelium in which growth arrest and differentiation start with detachment from the basal layer. On the other hand, intestinal epithelial cells carry out their entire renewal process while attached to a basement membrane and thus are not expected to require additional signals generated by cell suspension.

Accumulation of p27 during oligodendrocyte differentiation *in vitro* raises the possibility that p27 is part of an intrinsic counting mechanism that determines when precursor cell proliferation stops and differentiation begins. A separate effector mechanism would arrest the cell cycle when the counting mechanism indicated that it was time [156]. Studies of oligodendrocyte differentiation in p27-deficient mice show that a p27-dependent mechanism of growth arrest affects

proliferation in early phases of gliogenesis. A p27-independent event leads to withdrawal from the cell cycle and differentiation [157]. Lack of p27 results in expansion of the oligodendrocyte progenitor pool due to increased proliferation in early stages of gliogenesis. A decrease of cyclin E levels in the brains of p27 null mice coincident with oligodendrocyte growth arrest may be responsible for resetting the proliferative potential to wild-type levels. Interestingly, the *Drosophila* gene, *dacapo,* a member of the p21/p27 family of CDK inhibitors, seems to function during *Drosophila* embryogenesis to achieve a precisely timed exit from the cell cycle [158]. Dacapo-deficient embryos delay the normal cell cycle exit during development; many cells complete an additional cycle and subsequently become quiescent. This phenotype suggests that a similar action of p27 may lead to the increased body size observed in p27 knockout mice.

Given the wide variety of differentiated cell types, it is not surprising that common signaling molecules are used in different ways to achieve the same outcome. However, the role of p27 during differentiation in various tissues may help explain why decreased levels of p27 in certain human cancers correlate with poorly differentiated tumors and suggest that strategies to increase the level of p27 may be useful in cancer therapy. The fact that there already exist several agents that can increase the expression of p27, including cAMP, rapamycin, interferon-γ, and interferon-β, suggests that this approach may be clinically feasible [97, 105–107].

Studies in p27-deficient mice indicate that p27 has a selective effect on the self-renewing, mitogen-driven cell cycle characteristics of stem and some progenitor cells and little impact on terminal differentiation [159]. Thus, p27-null mice exhibit an expansion of hematopoietic progenitor cells without any defect in the differentiation capacity of these cells. Similarly, clinical observations in chronic myeloid leukemia patients have shown that immature myeloid cells are increased in chronic phase while their capacity to differentiate is retained.

CELL–CELL ADHESION

Malignant cells are generally anchorage independent. Anchorage independence is thought to be of fundamental importance for invasiveness and metastasis of tumors [160]. Early on, it was shown that the level and activity of p27 are elevated upon cell–cell contact of nontransformed fibroblasts or epithelial cells [1, 97, 161–163]. This suggests a direct role for p27 in intercellular adhesion. Two lines of evidence have supported such a role for p27 in intercellular adhesion, one via integrin signaling and the other via the Wnt pathway.

Cell adhesion to extracellular matrix (ECM) is mediated by binding of cell surface integrin receptors that both activate intracellular signaling cascades and mediate tension-dependent changes in cell shape and cytoskeletal structure to promote growth. S. Hang and colleagues showed that cell shape and cytoskeletal tension control p27 expression and cell cycle progression. Human capillary endothelial cells were prevented from spreading on ECM-coated adhesive islands and treated with mitogens. These cells, although exhibiting normal activation of the MAPK pathway, failed to progress through G1 and enter S phase. This cell cycle arrest correlated with a failure to increase cyclin D1 protein levels, downregulate p27, and phosphorylate pRb in late G1 [164].

E-cadherin is a major calcium-dependent homophilic cell adhesion molecule found on normal epithelial cells. Its intracellular domain binds directly to β-catenin which together with α-cadherin links E-cadherin to the actin cytoskeleton [165]. In addition to its role in adhesion, β-catenin has been implicated in the Wnt signaling pathway and interacts with the APC tumor suppressor protein as well as transcription factors of the LEF/TCF family [166–170]. In many carcinomas, cadherins are lost or downregulated, resulting in a reduced level of intercellular adhesion [171, 172] and cellular growth [173–178]. St. Croix *et al.* showed that E-cadherin can elevate p27 through inhibition of mitogenic signaling pathways initiated from receptor tyrosine kinases such as the epidermal growth factor receptor [179]. Thus, E-cadherin is not only an invasion repressor [180] but also suppresses proliferation by its ability to prevent growth-factor-induced reduction of p27 protein levels. Likewise, synthetic beads coated with N-cadherin block S-phase entry in N-cadherin-transfected CHO cells and in fibroblastic lines by increasing p27 protein levels [181].

Cancer cell lines often lose the ability to arrest growth in confluent two-dimensional cultures but become contact inhibited in three-dimensional cultures. We and others have found that p27 levels increase when cancer cell lines derived from breast, colon, ovaries, or lung are transferred from two- to three-dimensional culture (3570, J. Philipp-Staheli, unpublished results). Also, treatment of mammary carcinoma cells with a p27 antisense construct results in loss of cell–cell contact and growth stimulation, but only in three-dimensional culture [179]. This suggests that low p27 expression in certain human cancers may promote increased cell growth and metastasis. However, normal fibroblasts from p27-deficient mice undergo normal contact inhibition, suggesting the existence of redundant pathways or cell-type-specific differences that may play a role. In fact, quiescent fibroblasts from p27-deficient mice have increased cyclinA/cdk2 activity while fibroblasts from the same animals can inactivate

their CDKs normally. Recent data suggest that $p21^{Cip1}$ and $p130^{Rb2}$, two other cell cycle inhibitory molecules, may compensate for the loss of p27 by binding cyclin/CDK complexes [182].

p27 is often found in the nucleus and cytoplasm of both normal and cancer cells, but has been reported to be more highly expressed in the cytoplasm of certain human tumor cells. Recent findings on the yeast homologue of CDKIs, Far1p, indicate that p27 may have specific functions in the cytoplasm regulating cell polarity and morphology, factors that also influence cell–cell adhesion. Far1p is a yeast CDKI that is required for pheromone-induced G1 arrest [183, 184]. Yeast-mating pheromones trigger a MAPK signal transduction pathway, which results in Far1p induction, cell cycle arrest, and changes in cell polarity and morphology [185]. Blondel and colleagues discovered that upon pheromone exposure, Far1p is required in the nucleus to arrest the cell cycle and in the cytoplasm to establish cell polarity [186]. Far1p, like p27, contains a functional NLS and is exported from the nucleus by a member of the exportin family in a manner dependent on the GTP-bound small GTPase Cdc 42. Cytoplasmic Far1 functions as an adaptor that targets cytoplasmic polarity establishment proteins to the heterotrimeric G protein to organize the actin cytoskeleton toward the incoming signal [187, 188]. Further, Far1p sequesters the guanine-nucleotide exchange factor for Cdc42, Cdc24, in the nucleus [189]. Upon bud emergence, Far1p is phosphorylated by active cyclin/cdk and degraded, releasing Cdc24 into the cytoplasm. In contrast, in response to pheromones the Far1p/Cdc24 complex is exported into the cytoplasm by exportin, a mechanism that ensures that Cdc24 is targeted to the site of receptor-associated heterotrimeric G-protein activation at the plasma membrane, thereby allowing polarization of the actin cytoskeleton along the morphogenetic gradient of pheromone. In the yeast system, degradation and cytoplasmic translocation of Far1 have the same effect on cell growth. This suggests that nuclear export or degradation of Far1p may ensure growth arrest following pheromone exposure whereas cytoplasmic transfer of p27 by a nucleocytoplasmic transporter may ensure proper cellular polarization and morphology of the cell. It is intriguing to think that p27 may serve similar functions in mammalian cells with JAB1 as a nucleocytoplasmic transport protein. Further research is warranted to understand whether cytoplasmically located p27 has a function in cell growth and adhesion in normal and tumor cells.

ONCOGENES AND TUMOR SUPPRESSOR GENES INVOLVED IN p27 REGULATION

Since p27 acts directly on the cell cycle machinery, it is not surprising that the proteins whose expression is most commonly altered in cancer have an effect on p27 expression. Brc–Abl kinase transforms cells of both fibroblast and hematopoietic origin *in vitro* [190–192] and is constitutively active in chronic myeloid leukemia (CML). Bcr–Abl kinase activity leads to downregulation of p27 via proteasomal degradation and prevents p27 induction by TGF-β as well as and serum and mitogen deprivation [193]. Brc–Abl constitutively activates several signaling pathways important for the regulation of proliferation, survival, and adhesion including RAS, PI3K, MYC, JAK/STAT, focal adhesion kinase (FAC), Src kinases (Hck and Lyn), ERK, JNK, and JUN [190, 194–201]. Most of these molecules are known as oncogenes in their own right and have the ability to downregulate p27 independent of Brc–Abl as discussed above. In particular, inhibition of PI3K by LY294002 decreases the Brc–Abl-induced downregulation of p27 to some extent suggesting that the PI3K pathway is partially responsible for the Brc–Abl-induced reduction of p27 protein [193]. By simultaneously targeting apoptotic and cell cycle regulatory pathways, constitutively active Brc–Abl forces the cell to divide under inappropriate conditions such as DNA damage or suboptimal growth factor concentrations.

The ErbB2 receptor tyrosine kinase is a protooncogene which when overexpressed in primary tumors is associated with more aggressive tumor phenotype and poor patient prognosis, similar to the correlation of low p27 with high tumor grade and decreased patient survival in a wide spectrum of human tumors [202]. Elevated ErbB2 receptor signaling in overexpressing tumor cells potentiates G1/S progression by impeding p27 association with cyclin E/CDK2 complexes [203] and as a secondary step leads to lower p27 protein levels [204]. ErbB2 overexpression also leads to upregulation of c-myc and D-cyclins [203]. Thus, ErbB2 overexpression can provide an additional level of p27 deregulation during tumor development by maintaining p27 sequestration proteins and increasing cyclin E/CDK2 activity.

The dual-specificity phosphatase PTEN has recently been identified as a tumor suppressor gene frequently mutated in human tumors. In a subset of thyroid primary carcinomas and tumor-derived cell lines, a striking correlation between PTEN expression and the level of p27 protein is observed [205]. Bruni and his colleagues conclude from their work that one key target of PTEN suppressor activity is p27. In fact, PTEN inhibits phosphorylation of AKT by PI3K leading to transcriptional activation of p27 as described above. Germline mutation of the *von Hippel–Lindau* tumor suppressor gene in humans is associated with predisposition to a variety of malignant and benign neoplasms. The *von Hippel–Lindau* tumor suppressor protein stabilizes p27 upon serum withdrawal leading to cell cycle arrest [206]. In *VHP*-negative cells p27 sta-

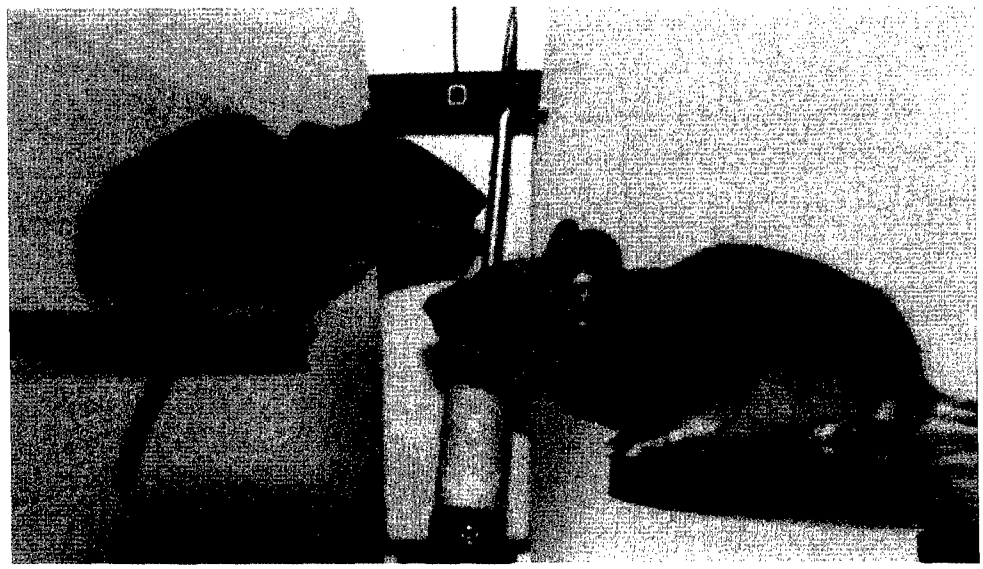

FIG. 1. *p27−/−* mice are larger than wild-type mice. The *p27−/−* mouse on the right weighs significantly more than its wild-type littermate shown on the left platform of the scales.

bilization does not occur and cells undergo G1–S transition although growth rates and p27 expression of *VHL*-negative and -positive cells under normal growth conditions are indistinguishable. It is striking that oncogenes and tumor suppressor genes both target p27, suggesting that p27 may be at a nodal point for controlling cancer cell disorder.

p27 TUMOR SUPPRESSION IN MICE

Three labs independently created p27 knockout mice using homologous recombination in ES cells and reported similar phenotypes [137, 159, 207]. *p27−/−* mice show increase growth rate and adults are 20–30% larger than wild-type littermates (Fig. 1). The increased size of the *p27−/−* animals is due to increased cellularity as opposed to increased cell size and points to a key role of p27 in controlling growth in all tissue compartments *in vivo*. Interestingly, tissues from *p27+/−* mice express roughly 50% of the normal level of p27 protein and these mice show an intermediate growth rate. This indicates that control of proliferation and even adult animal size is extremely sensitive to the levels of p27 protein. In addition, p27-deficient mice exhibit hyperplasia of the pituitary intermediate lobe and nearly 100% of mice (on a 129/Sv genetic background) eventually die as a result of benign pituitary tumors [159]. However, *p27−/−* mice do not spontaneously develop other tumor types.

ROLE OF p27 IN MULTISTEP CANCER PROGRESSION

As cancer is a multistep process requiring several genetic events, a lack of spontaneous tumor predisposition in p27-deficient mice is insufficient evidence to rule out a tumor-suppressing role for p27. Other genetic events may be required to elicit the latent tumor suppressing effects of p27. Indeed when p27-deficient mice are challenged with either the point mutagen ENU or the broad-spectrum mutagenic-agent-ionizing radiation, they show tumor predisposition in multiple tissues [36].

For example, in a cohort challenged with a single dose of 1 Gy radiation, the median tumor-free survival was reduced from >70 weeks in wild-type control mice to ~40 weeks in *p27−/−* mice. In addition to reduced tumor-free survival, p27-deficient mice show increased tumor multiplicity in diverse sites including small and large intestine, lung, ovary, uterus, and adrenal gland. *p27+/−* mice show an intermediate susceptibility, both in terms of tumor free survival and in terms of tumor multiplicity. Genetic and biochemical analysis of tumors from the latter group of mice reveals the unusual finding that the wild-type *p27* allele is not mutated and protein expression is not silenced in *p27+/−* tumors. These results differ from those obtained in other murine models of tumor suppressor knockouts. For example, tumors from *p53+/−*, *Rb+/−*, *p19/Arf+/−*, *Apc+/−*, *PTEN+/−*, and *Nf1+/−* mice all show frequent mutation or loss of the remaining wild-type allele [208–214], consistent with Kundson's two-hit model [215]. In these cases the tumor suppressor is recessive at the cellular level: complete loss of both alleles provides a much greater selective advantage for the cell than loss of a single allele. This genetic behavior is also observed in human tumors where biallelic mutations in these tumor suppressor genes are fre-

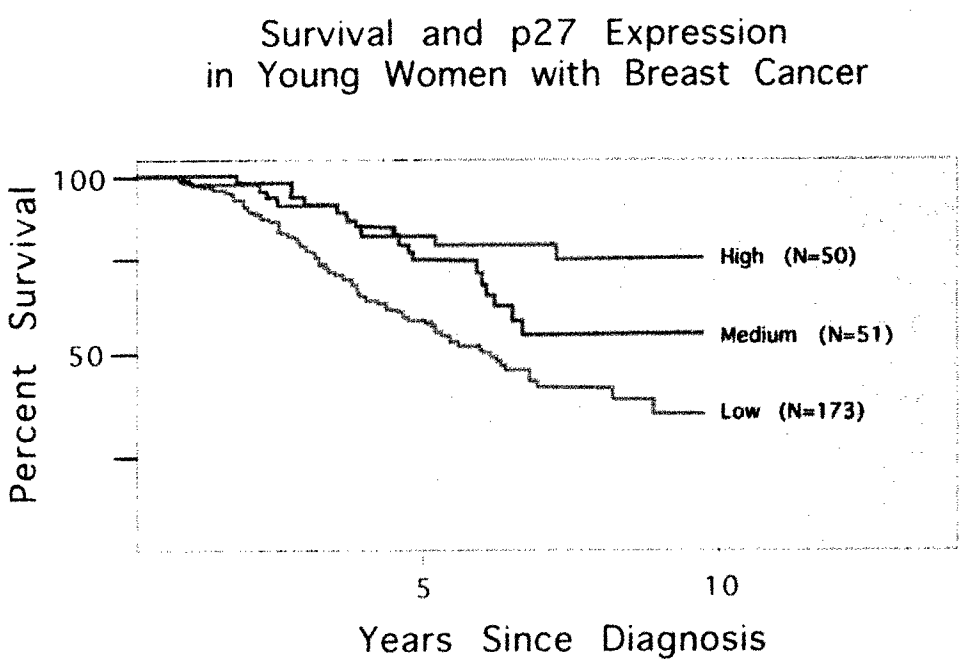

FIG. 2. Survival and p27 expression in young women with breast cancer. The Kaplan–Meier plot shows the association between survival and p27 expression in female breast cancer patients diagnosed at ages 20 to 44 years. Levels of staining for p27 were assigned on a scale from 0 to 6, with 0 indicating no p27 expression. The blue line represents those patients with high p27 expression (scores 4 to 6). The red line represents intermediate p27 expression (scores 2 to 3). The green line represents low p27 expression (scores 0 to 1), the vast majority of the breast cancer cases studied. The Kaplan–Meier plot is drawn from data initially published in [226].

quently seen. In contrast, biallelic mutations in *p27* are rarely seen in either human or murine cancer. The murine data unequivocally show a strong selective advantage to tumor development with loss of a single *p27* allele which results in 50% reduced protein level [36]. Why does p27 behave differently? The data indicate that inhibition of growth [159] and tumor development [36] are highly sensitive to absolute levels of p27 protein. Thus, halving the normal amount of p27 is sufficient to result in unchecked growth. There may not be a threshold for its activity but rather a signal–response continuum. At a biochemical level, this could be due to the stoichiometry of CDK inhibition by p27 [6]. From a teleologic standpoint, control of proliferation and body size has to be both sensitive and precise. This may be achieved by precisely controlling the levels of p27 and the ensuing proliferative response according to cell-type requirements. An all or nothing response would not permit such tight control.

The development of a tumor is an evolutionary process that requires multiple genetic and phenotypic changes. What is the specific role of p27 in this process? We previously used a two-stage skin tumor model to evaluate at what stage p27 influences cancer progression. Treatment of the dorsal skin of mice with a single dose of DMBA followed by repeated applications of the tumor promoter TPA results in benign papillomas which stochastically progress to malignant carcinomas. Mutation of the H-*ras* oncogene is an initiating event in this system and the majority of tumors contain the identical codon 61 activating mutation [216–218]. As signaling from H-ras leads to an increase in cyclin D and cell proliferation, p27 could play a role in counteracting ras signaling. Skin tumors from *p27−/−* mice uniformly contain the same activating point mutation in the H-*ras* oncogene, as do skin tumors from wild-type mice [219]. This indicates that p27 deficiency does not functionally substitute for mutant ras. It also suggests that p27 may normally counteract the proliferative effect of mutant ras: in the absence of p27, tumors with mutant ras grow faster. However, p27-null mice do not display an increase in the total numbers of papillomas, suggesting that the mechanism of tumor suppression by p27 involves increased tumor growth but is unrelated to tumor initiation by DMBA treatment. This study also shows that lack of p27 leads to a slight increase in malignant progression. Similar to observations from human cancer, a subset of skin tumors from wild-type mice show a reduction of p27 expression. Furthermore, high-grade tumors tend to have lower p27 expression than more differentiated tumors. Similarly, treatment of mouse skin with TPA and other tumor promoters reduce p27 expression in basal epithelial cells suggesting one mechanism of action of tumor promoters is to decrease p27 thus affecting the hyperproliferative response. Whether the de-

crease of p27 in quiescent basal keratinocytes after promoter treatment or low p27 in murine skin carcinomas is causal for increased proliferation and tumor progression still needs to be elucidated. However, these data support the idea that p27 may have a general role in suppressing postinitiation clonal expansion of initiated, hyperplastic, or benign cells.

Reduction of p27 cooperates with mutation in two other cell-cycle-related genes. Pituitary and thyroid C cell tumorigenesis is accelerated in *Rb+/− p27−/−* compound mutant mice [220] and pituitary tumorigenesis is accelerated in $p18^{INK4c}-/-$ *p27−/−* compound mutant mice [221]. Furthermore, in combination with transgenic mice expressing human growth-hormone-releasing hormone, p27-deficient mice show earlier appearance and increased penetrance of pituitary tumors [222]. Thus, p27 inhibits tumor growth in response to multiple oncologic signals and in multiple tissues. The diversity of physiological and exogenous signals that impinge and act upon p27 and the divergent array of human tumors that show alterations in p27 expression suggest that p27 may be involved in yet more pathways. If p27 plays a key role in cell fate decisions as accumulating data suggest, then p27 would be a major rate-limiting factor for tumor progression.

p27 AND HUMAN CANCER PROGNOSIS

A number of recent studies have demonstrated the prognostic significance of p27 in human cancer. Reduction of p27 has been observed to varying degrees in all human malignancies examined to date [reviewed in 19, 223]. Decreased levels of total p27 protein are associated with high tumor grade and stage in human breast, colorectal, and gastric cancer, among others [23, 27, 30, 224, 225]. The number of human cancers in which disease-free survival is correlated with p27 expression is increasing at a rapid rate. Reduction of p27 correlates significantly with decreased survival in breast (Fig. 2), colorectal, gastric, ovary, prostate, bladder transitional cell, and esophageal squamous cell carcinoma patients, among others [23, 24, 29, 226–233]. These global findings regarding p27 expression in human cancer are significant, as it is highly unusual for a single molecular marker to have strong prognostic value in such a wide array of tumor types. Perhaps relevant to the decreased survival for patients with reduced p27 is the observation that reduction of p27 is associated with the development of colorectal carcinoma metastases [234]. Overwhelmingly, the evidence indicates that p27 plays a critical role as a tumor suppressor for multiple human cancers.

Clearly, p27 is a strong prognostic marker in numerous epithelially derived human cancers. The "Holy Grail" of cancer molecular biology is to provide a cancer patient with individually tailored therapy based on the specific molecular fingerprint of his or her tumor. Recent work indicates that p27 has prognostic significance not only with respect to disease survival, but is also predictive of response to specific chemotherapeutic regimens. In a study of 77 patients with squamous cell carcinoma of the cervix before and after radiotherapy, high p27 expression prior to radiotherapy predicted increased survival following radiotherapy [235]. In a separate study of 22 patients with recurrent non-small cell lung carcinoma, high (>50%) p27 expression predicted increased survival after platinum-based chemotherapy [236]. Future work should address chemotherapeutic response based on p27 expression level in other cancers as well.

THE FUTURE

The role of p27 in cell cycle inhibition has been increasingly well defined over the past years. Several p27 attributes suggest potential roles for p27 in tumorigenesis. In general, tumor suppressor genes lead to an increase in p27 whereas oncogenes lead to its downregulation. The complex interaction of molecules regulating nuclear import and export and availability and degradation of p27 facilitates fine tuning of the abundance of p27 molecules that are free to interact with and inhibit cyclinE/CDK2 and block cell cycle progression. Any of the molecules impacting p27 levels in the nucleus might be a target for alterations in cancer. The central role of p27 in cellular growth is further underscored by its ability to mediate responses from growth factors, hormones, interleukins, factors that trigger differentiation, and cell–cell adhesion molecules alike. It will be important to identify which of these responses are impaired in cancer cells with low p27. Further, as opposed to p27's role in the cell cycle, its role in differentiation, cell–cell adhesion, and apoptosis is less well defined.

p27 has been widely implicated as a prognostic marker in human tumors. To be of real clinical value, a marker must fulfill three criteria: (i) provide reproducible results, (ii) provide diagnostic information independent from other diagnostic and pathological criteria, and (iii) provide information that would justify alteration of the treatment protocol. Although p27 clearly fulfills the first two criteria, additional knowledge is required to understand how p27 levels in human tumors can be altered by treatment. An additional problem is that most human tumor types displaying low p27 levels lack reliable curative therapies. However, promising results indicate that p27 may be associated with survival in patients after radiotherapy and removal of a prostate tumor [237]. Therefore, a thorough understanding of the regulation of p27 in these tissues and its misregulation in cancer should stimulate therapeutic innovation.

p27-deficient mice have shown preferential tumor susceptibility in tissues of epithelial origin. This is of interest, since 90% of human cancers are epithelial in origin. Other knockout mice deficient in global tumor suppressor genes such as *Rb, p53, p16,* and *p19/Arf* are primarily predisposed to tumors of the pituitary, hematopoetic, or mesenchymal tissues and less often to tumors of epithelial origin [238] detracting from their usefulness as models of human cancer. With further development, it is probable that p27-deficient mice will complement existing models to address critical questions regarding cancer etiology, prevention, and therapy. p27-deficient mice may play a major role in testing environmental chemicals for carcinogenic activity specifically in epithelial tumors, for discerning the role of p27 in therapy and prognosis, and for testing novel dietary or therapeutic interventions.

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